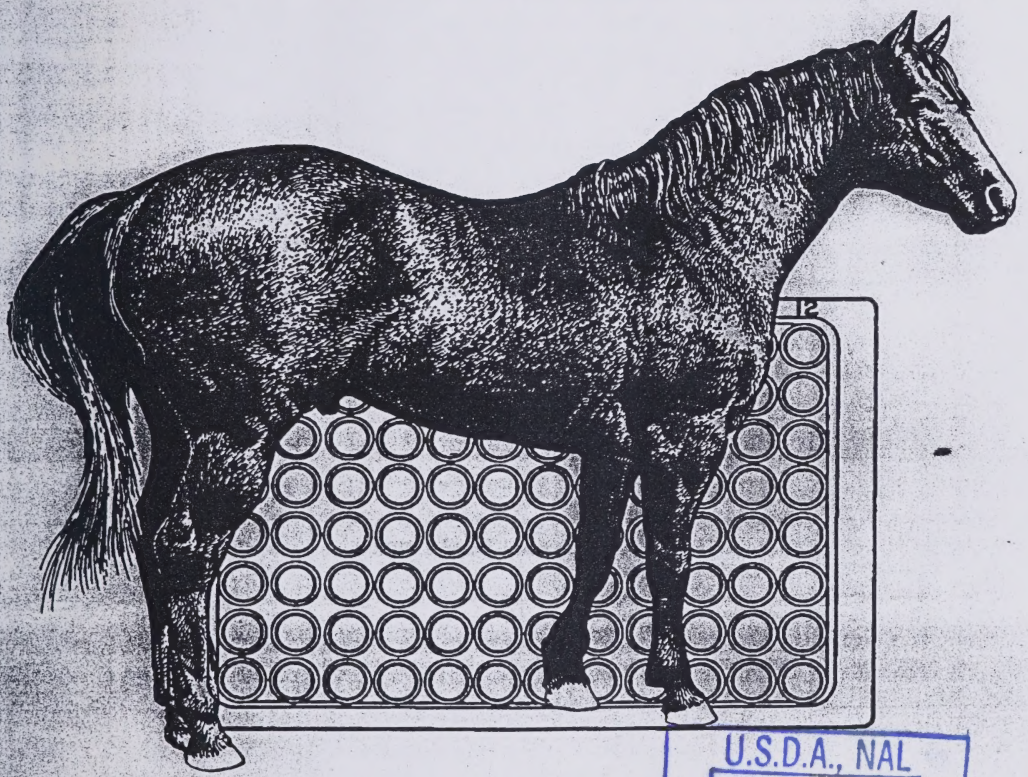
DAVID P. OLSON, D.V.M., Ph.D.

Piroplasmosis, Dourine, and Glanders Complement Fixation Test-Micro Method

Revised June 1992



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Ames, Iowa

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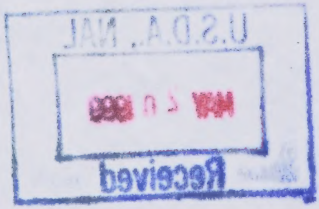

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I. Preparation of Veronal Buffered Diluent (VBD)

A. Add 200 ml of stock buffer to a 1-liter graduated cylinder. (See Appendix - Preparing Stock Buffer Solution).

B. Fill the cylinder to the 990 ml mark with distilled water, and pour into a flask.

C. Add 10 ml of 10% gelatin (10 gm of dry gelatin dissolved by boiling in 100 ml distilled water). This will yield a final concentration of 0.1%. Mix thoroughly by inverting or with a magnetic stirrer.

D. Check the pH of the VBD. If the pH is below 7.3 or above 7.4, discard the VBD, and repeat preparation.

E. Label the VBD including date of preparation.

F. Store the VBD in the refrigerator.

II. Preparation and Standardization of Sheep Erythrocyte Suspension

A. Preparation and Washing of Sheep Erythrocytes

1. Determine the volume of packed sheep erythrocytes required for the 2.0% red blood cell (RBC) suspension.

a. For the preparation of color standards, 6.0 ml of 2.0% RBC's are needed.

b. Determine whether a hemolysin titration is necessary. (An hemolysin titration should be performed each time a new lot of 1:100 hemolysin solution is prepared or a new lot of sheep RBC's is used. If so, increase the volume of 2.0% RBC's needed by 9.0 ml).

c. For the complement titration, increase the volume of 2.0% RBC's needed by 6.0 ml.

d. If diagnostic tests are to be done, determine the volume of 2.0% RBC's required:

1) Add 2.5 ml for each 16 serums to be screened.

2) Add 2.5 ml for each 8 serums to be titered.

e. Find the total volume of 2.0% RBC's required by adding the volumes determined in Steps a-d above.

f. Determine the volume of packed RBC's required by setting up a proportion:

$$\frac{2.0}{100} = \frac{X^*}{\text{Number of ml of 2.0\% RBC;s needed}}$$

* X = Number of ml of packed RBC's needed

2. To determine the volume of preserved blood necessary to provide the required volume of packed RBC's, multiply the required volume of packed RBC's by 10 (preservative - Alsever's Solution - see appendix).
3. Filter the preserved blood through two layers of sterile gauze into round or conical bottomed 50 ml centrifuge tubes.
4. Centrifuge the blood at 900 x g[a] for 10 minutes.
5. Remove the supernatant fluid by suction.
6. Add cold VBD to fill the tube (9 parts VBD - 1 part packed RBC).
7. Mix gently by inverting tube to resuspend the cells and centrifuge at 900 x g for 10 minutes.
8. Carefully remove the supernatant fluid and white blood cell layer by suction.
9. Add cold VBD to fill the tube.
10. Mix gently by inverting tube to resuspend the cells and centrifuge at 900 x g for 10 minutes.
11. Look at the supernatant fluid to see whether it is colorless. If not, the cells are too fragile and should be discarded. Obtain new preserved blood, and return to Step 2.
12. Remove the supernatant fluid by suction.
13. Add cold VBD to fill the tube.
14. Mix gently by inverting tube.
15. Centrifuge at 900 x g for 10 minutes.

[a] Obtained by centrifugation at 2,000 rpm in a SB 1 International Centrifuge with a head having a radius of 13 cm. or at 1,700 rpm in a SB 2 International Centrifuge with a head having a radius of 19 cm. The general case formula for obtaining the number times gravity is 1118 x R x (rpm)2 x 10-8 (R = radius of centrifuge head in cm.).

16. Carefully remove as much supernatant fluid as possible without disturbing the cells.
17. For each 35 ml of cell suspension needed, suspend 1 ml of packed cells in 34 ml of VBD. Use an appropriate sized Erlenmeyer flask.

B. Standardization of 2.0% RBC's - Spectrophotometric Method

1. Turn on the spectrophotometer to allow for adequate warm-up time.
2. Swirl the flask containing RBC's gently to secure an even suspension.
3. Carefully pipette 1.0 ml of the 2.0% suspension into a 25 ml volumetric flask.
4. Fill the flask to the 25 ml mark with cyanmethemoglobin reagent. (See appendix for cyanmethemoglobin preparation).
5. Mix well by inverting the flask at least 10 times.
6. Allow the suspension to stand at least 20 minutes at room temperature. While the suspension is standing, you should check the 40% cyanmethemoglobin standard to determine whether the optical density (OD) reading is within + 3% of the original reading. If not, prepare new standards, and calculate the target OD. (If cyanmethemoglobin standards have not been prepared previously, factor and target OD must be determined - see appendix).
7. When at least 20 minutes are up, mix the suspension again by inverting the flask.
8. Select a clean, calibrated cuvette, and fill it with the sample; wipe the cuvette with a paper tissue to remove fingerprints.
9. Read the OD of the sample against the reagent blank (0 mg%) at 540 nm being careful to read the OD (absorbance-logarithmic) scale. This is the test OD.

10. Calculate the final volume of the desired 2.0% suspension using the formula:

$$\text{Final volume} = \frac{(\text{OD of test Suspension}) \times (\text{original volume of test suspension} - 1.0\ \text{ml})}{\text{Target OD for a 2.0\% suspension}}$$

11. Dilute the suspension with cold VBD to the desired final volume.

III. Hemolysin Titration[a]

A. Preparation of 1:100 Hemolysin Dilution

1. Add 9.0 ml of cold VBD to the flask, and mix by swirling.
2. Add 2.0 ml of glycerinized hemolysin, and mix by swirling.
3. Store the 1:100 hemolysin dilution in a refrigerator.

B. Preparing 1:1000 Hemolysin Dilution

1. Label a 20 x 125 mm tube for the 1:1000 hemolysin dilution.
2. Add 18 ml of cold VBD to the tube.
3. Add 2 ml of 1:100 hemolysin dilution and mix.

C. Preparing Further Hemolysin Dilutions

1. Label eight 20 x 125 mm tubes with the final hemolysin dilutions shown in the first column of Table 1.
2. Use a 5.0 ml pipette to add the volumes of VBD shown in Table 1 to the tubes.
3. Use a 5.0 ml pipette to add the quantities of 1:1000 hemolysin dilution shown in Table 1 to the tubes.

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[a] An hemolysin titration should be performed each time a new lot of hemolysin is used.

Table 1. Preparation of Further Hemolysin Dilutions

Final Hemolysin Dilution	VBD, ml	1:1000 Hemolysin Dilution, ml
1:1500	1.0	2.0
1:2000	2.0	2.0
1:2500	3.0	2.0
1:3000	2.0	1.0
1:4000	3.0	1.0
1:8000	7.0	1.0
1:16,000	15.0	1.0
1:32,000	15.5	0.5

D. Preparing 1:250[a] Dilution of Complement (C′)

1. Measure out 18 ml of cold VBD, and pour into a test tube (20 X 125 mm - screwcapped or similar).
2. Obtain undiluted C′, and place it in an ice bath.
3. Draw up undiluted C′ in a 2.0 ml pipette to beyond the 0 mark. Wipe the tip of the pipette, and return the excess above the 0 mark to the stock container.
4. Deliver 2.0 ml of C′ dropwise into the VBD in the test tube. Mix by swirling the tube gently. (1:10).
5. Measure out 7.2 ml of cold VBD, and put it in a test tube (20 x 125 mm - screwcapped), and add 0.3 ml of dilute C′ (1:10) using a 1.0 ml pipette. (1:250). Prepare C′ dilutions 1:200 and 1:300.
6. Mix by tipping the test tube gently.
7. Place the diluted C′ in the refrigerator for at least 20 minutes. (Complement diluted greater than 1:10 should be used within 2 hours). During this 20-minute waiting period, continue with E1.

E. Preparing Sensitized RBC′s for Hemolysin Titration

1. Place nine 13 x 100 mm tubes in the rack. Label the first tube 1:1000, label the remaining tubes with the final hemolysin dilutions shown in Table 1.
2. Add 2.0 ml of the standardized 2.0% sheep RBC suspension to each of the 9 tubes (prepared II B 11).
3. Add slowly, with constant swirling, 2.0 ml of the 1:1000 hemolysin dilution to the sheep RBC′s in the 1:1000 tube.
4. Mix the 1:1500 hemolysin dilution with a pipette and add, with constant swirling, 2.0 ml of the dilution to the sheep RBC′s in the 1:1500 tube.

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[a] Commercial complement diluted 1:250 generally will yield 30% to 80% hemolysis for optimally sensitized cells. To obtain the correct percent hemolysis with a less active complement, it may be necessary to use a 1:200 dilution for the titrations. With a very potent complement, it may be necessary to use a 1:300 dilution.

5. Shake the rack for mixing, and incubate the 9 tubes in a 37 C water bath for 10 minutes to sensitize the cells. While the tubes are incubating, continue with Step F1.

F. Setting up Hemolysin Titration

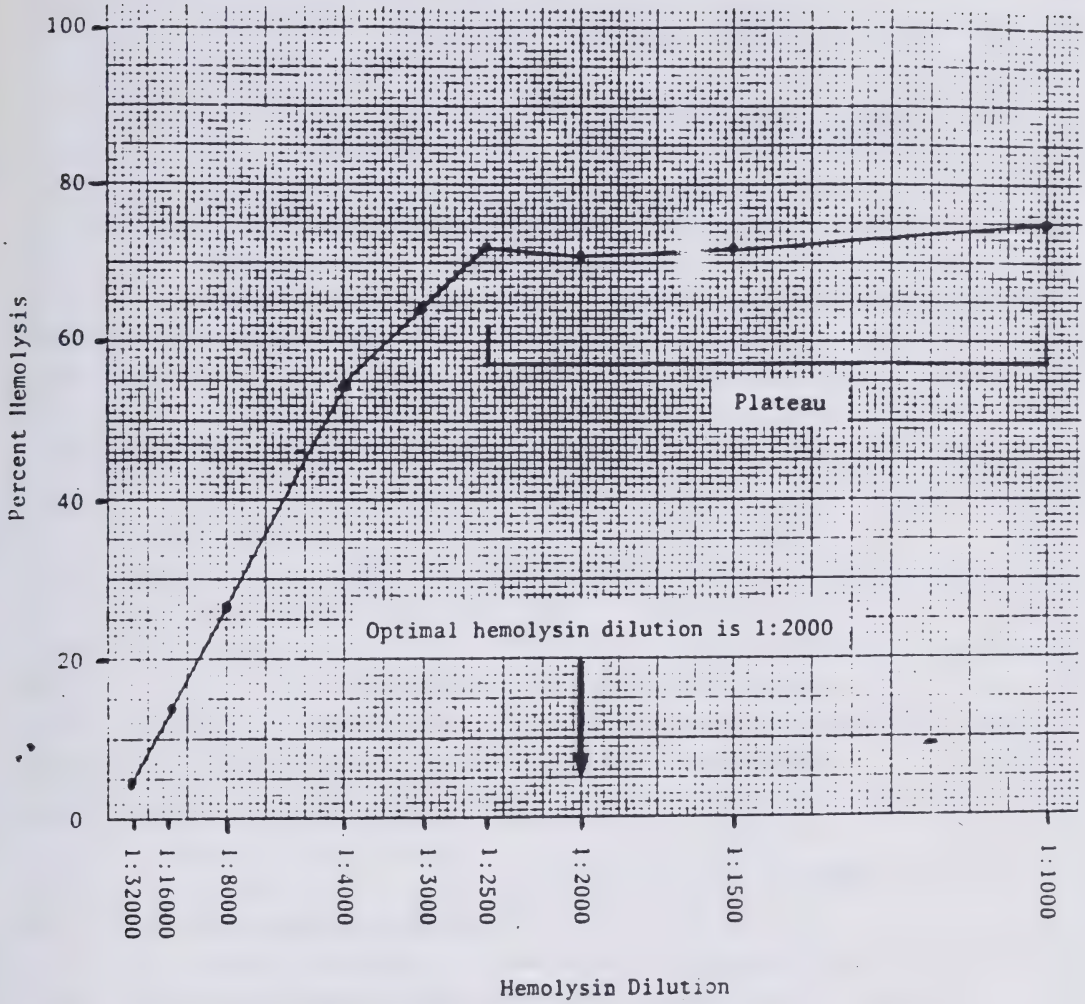
1. Label three sets of 9 serologic tubes (13 x 100 mm) with the following hemolysin dilutions: 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:4000, 1:8000, 1:16,000, and 1:32,000. Label one set for each C´ dilution.
2. Add 0.8 ml of cold VBD to each tube.
3. Add 0.4 ml of the dilute complement to each of the 9 tubes (prepared in Steps D1-D7 one set for each C´ dilution).
4. Shake the rack for mixing.
5. Add 0.8 ml of RBC´s sensitized with the same hemolysin dilution as the label on the tube to each tube (prepared Step E1-E6).
6. Mix each tube by shaking.
7. Incubate the tubes in a 37 C water bath for 30 minutes shaking once after the first 15 minutes of incubation.
8. While the tubes are incubating, prepare hemoglobin color standards. (See appendix for procedure).

G. Determining Hemolysin Dilution Needed for Sensitization of 2.0% RBC´s

1. Centrifuge the tubes at 900 x g for 10 minutes.
2. Compare each tube with the hemoglobin color standards (prepared in Step F8.
    a. If the tube matches a standard, read and record the percent hemolysis.
    b. If the tube does not match a standard, interpolate to the nearest 5%, and record the reading.
3. Plot on ordinary arithmetic (linear) graph paper the amount of hemolysis obtained with each dilution of hemolysin. (See Figure 1).
4. Draw a line through the points plotted.
5. Examine the graph for a "plateau," that is, the level at which increasing the amount of hemolysin produces no marked increase in percent hemolysis. (See Figure 1).

Figure 1

Hemolysin titration



*To prepare the hemolysin dilution scale; let the left end of the scale be 0, and lay off a suitable length for the 1:1000 dilution. Other dilutions are represented as fractions of this length. Thus 1:1500 = 2/3 of 1:1000, 1:2000 = 1/2 of 1:1000, 1:2500 = 2/5 of 1:1000, 1:3000 = 1/3 of 1:1000, 1:4000 = 1/4 of 1:1000, 1:8000 = 1/8 of 1:1000, 1:16000 = 1/16 of 1:1000 and 1:32000 = 1/32 of 1:1000. (Arithmetic graph paper, 20 X 20 to the inch is most suitable, using a 6-inch horizontal distance for the 1:1000 dilution).

6. Read the second dilution on the plateau as the hemolysin dilution to be used for subsequent RBC sensitization (optimal hemolysin dilution).

IV. Complement Titration[a]

A. Preparing Complement

1. Measure out 18 ml of cold VBD, and pour it in a test tube (20 x 125 mm - screwcapped or similar).
2. Obtain undiluted C′, and place it in an ice bath.
3. Draw up undiluted C′ in a 2.0 ml pipette to beyond the 0 mark. Wipe the tip of the pipette, and return the excess above the 0 mark to the stock container.
4. Deliver 2.0 ml of C′ dropwise into the VBD in the test tube. Mix by swirling the tube gently. (1:10).
5. Measure out 14.7 ml of cold VBD, put in a test tube (20 x 125 mm - screwcapped), and add 0.3 ml of C′ 1:10) using a 1.0 ml pipette. (1-500). Prepare C′ dilutions 1:400 and 1:600.
6. Mix by tipping the test tube gently.
7. Place the diluted C′ in the refrigerator for at least 20 minutes. (Complement diluted greater than 1:10 should be used within 2 hours). During this 20-minute waiting period, continue with B1.

B. Preparing Sensitized RBC′s

1. Add 3 ml of standardized 2.0% RBC suspension to a 20 x 125 mm tube (prepared II B 11).
2. Prepare optimal hemolysin dilution (III G 6) from the 1:100 stock hemolysin solution.
3. Add 3 ml of optimal hemolysin dilution to the RBC′s with rapid swirling.
4. Incubate for 10 minutes in a 37 C water bath. While the RBC′s are incubating, continue with Step C1 below.

[a] Complement must be titrated each time a new lot of sheep RBC′s is used.

C. Setting Up Complement Titration

1. Label 3 sets of serologic tubes (13 x 100 mm) 1-4. Label one set with each C′ dilution.
2. Add VBD in the amounts shown in Table 2 to each set of tubes.
3. Add C′ dilution in amounts shown in Table 2 to appropriate tube set.
4. Add 0.4 ml of sensitized RBC′s to each tube (prepared in Step B4).
5. Shake the rack for mixing, and place it in a 37 C water bath for 15 minutes.
6. When 15 minutes are up, remove the rack, and shake to resuspend unlysed RBC′s.
7. Return the rack to the water bath for an additional 15 minutes to give a total of 30 minutes of incubation.

Table 2. Complement Titration

	1	2	3	4
VBD	0.3[a]	0.2	0.1	0.0
Dilution of C′	0.3	0.4	0.5	0.6
Sensitized RBC′s	0.4	0.4	0.4	0.4

D. Reading Percent Hemolysis

1. Remove the rack from the water bath, and centrifuge the tubes at 900 x g for 10 minutes.[b]
2. Compare each tube in a set with the hemoglobin color standards. If the tube matches a standard, read and record the percent

[a] Some spectrophometers require a greater volume - volumes may be doubled. All volumes are in milliliters.

[b] See footnote on page 2 for the general case formula for obtaining the number times gravity.

hemolysis. If the tube does not match a standard, interpolate to the nearest 5%, and record the reading (comparisons may be made with a spectrophotometer at a wavelength of 540 nm).

3. Read and record the percent hemolysis for each set.
4. For each of the four tubes (in each titration), plot on probability by logarithmic 2 cycle log graph paper the volume of the 1:500 dilution of C′ in ml against the corresponding percent of hemolysis. Plot volume of C′ for the other two dilutions of C′ on graph paper. (See Figure 2).
5. Examine each graph to see whether two of the points fall on the left side of the vertical "50" line and two on the right. If so, continue with Step 7. If more than two points fall on the left side of the vertical "50" line, use the C′ titration with a dilution of C′ lower than 1:500 (1:400). If more than two points fall on the right side, use the C′ titration with a dilution of C′ higher than 1:500 (1:600).
6. Join the two points plotted for tubes 1 and 2, and mark the midpoint of the line joining them.
7. Join the two points plotted for tubes 3 and 4, and mark the midpoint of the line joining them.
8. Draw a line between the two midpoints. (See Figure 2).
9. Determine the slope of the line which joins the two midpoints:
    a. From any point near the left end of the line joining the two midpoints, measure horizontally to a point 100 mm to the right.
    b. Measure the vertical distance in millimeters from that point upward to the line joining the two midpoints.
    c. Divide the vertical distance by 100 mm to obtain the slope. If the slope is 0.44 ± 20%, continue with Step E1 below. If the slope is not within ± 20%, repeat the C′ titration. (Reproducible results are obtained only when the slope of this line falls within ± 20% of 0.44).

E. Determining the Dilution of C′ Needed for Diagnostic Test

1. From the intersection of the vertical "50%" line with the line joining the two midpoints, draw a dotted horizontal line to the vertical axis on the left. (See Figure 2).
2. Read the volume in ml of the 1:500 dilution of C′. This volume contains one 50% hemolytic unit of C′ ($C'H_{50}$). Since volumes were doubled in Table 2, the volume must be divided by 2 to determine volume containing one $C'H_{50}$.
3. Determine the volume containing 5.5 $C'H_{50}$ by multiplying the volume containing one $C'H_{50}$ by 5.5. (5.5 $C'H_{50}$ in 0.2 ml is the quantity required for diagnostic test).
4. Calculate the dilution of C′ necessary to obtain 5.5 $C'H_{50}$ in 0.2 ml using the following equation:

$$\frac{\text{Dilution of C′ used in titration}}{\text{Volume containing 5.5 } C'H_{50}} = \frac{X^*}{0.2}$$

* X = dilution of C′ needed for 5.5 $C'H_{50}$

Example: The volume containing 5.5 $C'H_{50}$ at 1:500 dilution is 2.31 ml (5.5 · 0.42 ml).

The dilution of C′ necessary to obtain 5.5 $C'H_{50}$ in 0.2 ml is calculated as follows:

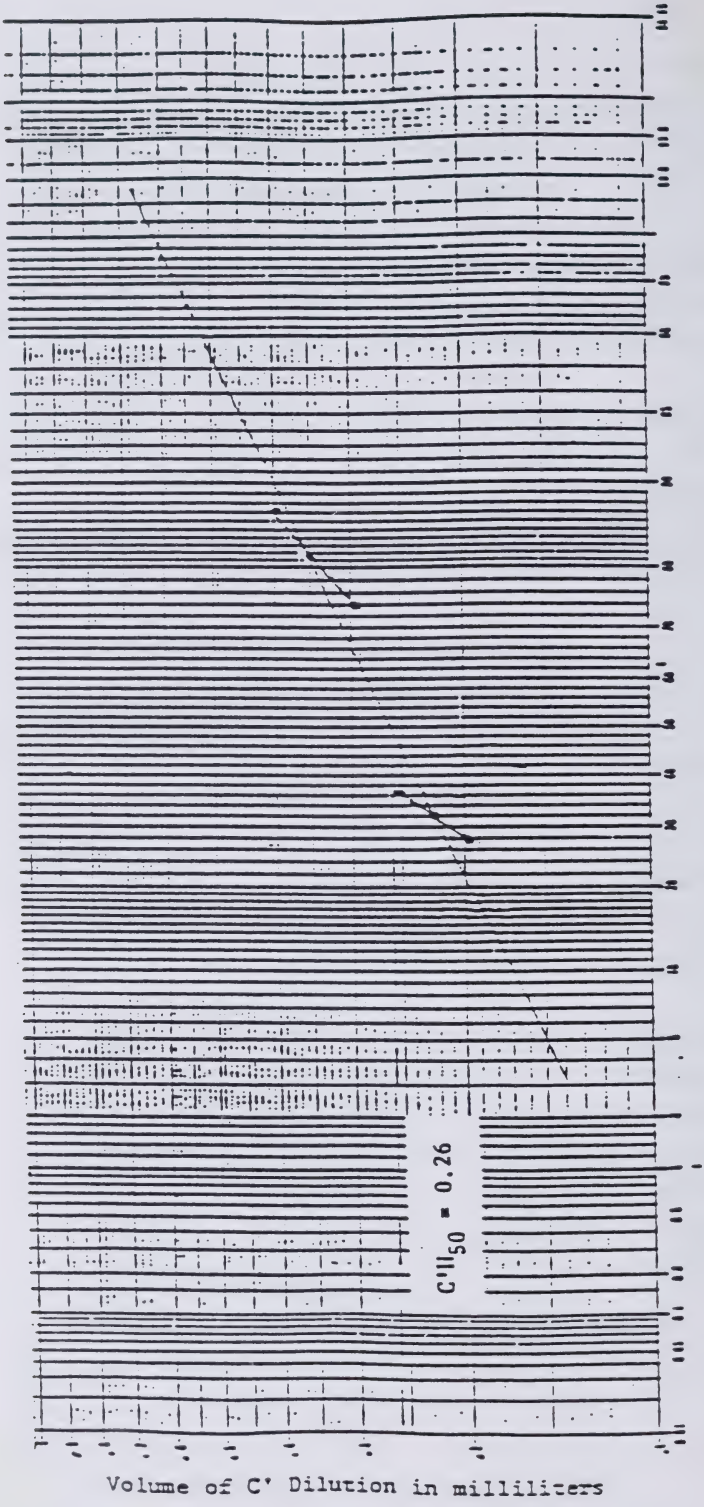
a. $\frac{500}{2.31} = \frac{X}{0.2}$

b. $2.31X = 500 \cdot 0.2 = 100$

c. $X = \frac{100}{2.31} = 43.3$

d. The dilution of C′ needed is 1:43.

Figure 2.
Complement titration



V. Diagnostic Test: Micro Method

A. Serum Inactivation and Plate Labeling

1. Inactivate diluted (0.1 ml serum plus 0.4 ml VBD-1:5) unknown serums, a known positive serum, and a known negative serum for 35 minutes in a 58 C water bath. During this incubation period, continue with Steps A2-3 and B1-3.
2. Screen test - label 2 wells on a plate for each serum in the test. Serum titration test - Label one row of 8 wells for each serum.
3. Number 9 wells on an additional plate for reagent controls according to Table 3.

B. Antigen Preparation

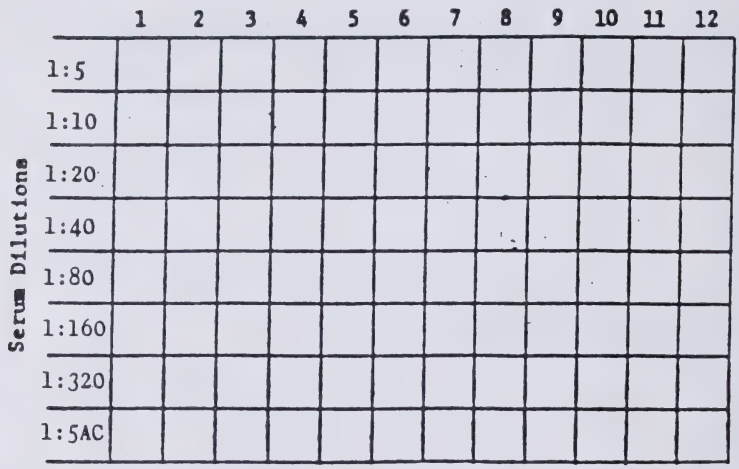
1. Determine the volume of test antigen required by multiplying the number of wells receiving test antigen by 0.025 ml. Allow excess for pipetting (3 ml).
2. Dilute test antigen to dilution stated on the label using cold VBD, and mix thoroughly.
3. Store the antigen at 0 C until needed.

C. Serum Dilution

1. When the serum has been in the water bath for 35 minutes (Step A1), remove and allow to cool to room temperature.
2. Use screen test (a) or titration test (b) as appropriate.
   a. Serum - Screen Test Only
      1. Using a 0.025 dropper pipette, transfer 0.025 ml of diluted serum to each of two wells - serum dilution complete.
      2. Add amount of VBD specified in Table 3 to the reagent control wells. Continue at Step D1.

b. Serum - Titration Test

1. Use a 0.025 ml dropper pipette to add 0.025 ml of cold VBD to wells B through G in a row for each serum being tested. (See Figure 3 below).



2. Add amounts of VBD specified in Table 3 to the reagent control wells.
3. Using 0.025 ml dropper pipette, transfer 0.025 ml of each diluted serum to the wells 1:5, 1:10, and AC. With 0.025 ml loop (microdiluter) mix dilute serum in well 1:10; twirl the loop rapidly for 4 seconds to mix. Then place the diluter in well 1:20, and continue the dilution process in each successive well through 1:320. Repeat for each serum.

D. Diluted Complement Preparation

1. Determine the volume of diluted C′ required for the test by multiplying the number of wells in the test by 0.025 ml. Allow some excess for pipetting (5 ml).
2. Calculate the volumes of VBD and C′ needed to prepare the required volume of dilute C′ containing 5.5 $C'H_{50}$ as determined in the C′ titration in tubes. (See appendix for formula).
3. Add the calculated volume of VBD to a small flask.

4. Add the calculated volume of 1:10 C′ dropwise to the VBD.
5. Mix gently to avoid foaming.
6. Allow the diluted C′ to stand at 0 C for at least 20 minutes. Continue with Step E1.

E. Reagent Addition and Incubation of Diagnostic Samples

1. Use a 0.025 ml dropper pipette to add 0.025 ml VBD to each AC well (well H for titration or first well for screen test). This is the serum anticomplementary (AC) well. (See Figure 3).
2. Use a 0.025 ml dropper pipette to add 0.025 ml of the diluted test antigen (Step B3) to wells labeled 1:5 through 1:320 or the titration test and to the reagent control wells designated in Table 3. Screen test - add to second well only.
3. Use a 0.025 ml dropper pipette to add 0.025 ml of diluted C′ containing 5.5 $C'H_{50}$ to wells A through H and to the reagent control wells specified in Table 3. Add to both wells for the screen test.
4. Dilute 1 ml of C′ with 1 ml of VBD. Use a 0.025 ml dropper pipette to add 0.025 ml of diluted C′ to the reagent control wells specified in Table 3.
5. Turn on mechanical vibrator before placing plates on the vibrator to mix. After mixing 1 minute, remove plates before turning off vibrator. This procedure should be followed each time plates are shaken.
6. Cover the plates to minimize evaporation, and incubate for 1 hour at 37 C. Do not stack plates. During the last 20 minutes of the incubation period, Steps E7 to E12 must be completed.
7. Prepare sensitized cells. Determine the volume of sensitized cells needed for the test by multiplying the total number of wells in the test by 0.05 ml and adding excess for pipetting (5 ml).
8. Remove the 2.0% standardized RBC suspension from the refrigerator, and shake it gently to secure an even suspension (Step II B11).

9. To a flask, add a volume of standardized cells equal to half the volume of sensitized cells needed.
10. Add an equal volume of optimal hemolysin dilution (Step III G 6) to the standardized cells with rapid swirling.
11. Incubate the cells in a 37 C water bath for 10 minutes.
12. Remove the sensitized cells from the water bath.
13. Use of 0.05 ml dropper pipette to add 0.05 ml of the sensitized cells to each test well and to the reagent control wells designated in Table 3. (Screen and Titration Tests).
14. Add 0.025 ml of 2% unsensitized RBC's to the reagent control wells designated in Table 3.
15. Use a 0.025 ml dropper pipette to add 5 drops of each hemoglobin color standard to individual wells on the plate containing the reagent controls.
16. Cover each plate with 3-inch transparent plastic or cellophane tape.
17. Shake the plates until the cells are suspended (approximately 1 minute.
18. Place the plates in a 37 C incubator for 20 minutes. Do not stack the plates.
19. Remove plates, and shake on shaker to resuspend unlysed cells. Return to incubator for an additional 25 minutes to give a total of 45 minutes incubation.

F. Reading and Recording Test Results

1. Centrifuge the plates for 5 minutes at 300 x g. If centrifuge carriers are not available, let plates stand in the refrigerator for 2 to 3 hours until the cells settle.
2. Read and record the results of reagent controls by comparing the percent hemolysis with the color standards. Interpret results based on Table 4.
3. Compare the reagent control readings with those in Table 3 to see whether they are acceptable. If the controls are not acceptable, disregard the test results, and repeat the test.

4. If the reagent control readings are acceptable, read and record the percent hemolysis in each well in the test. Use the signs shown in Table 4 for recording the readings.
5. The RBC's should be completely hemolyzed in the AC control well (1:5 dilution). If not, this serum is AC, and the test results are unreliable. Record this serum as being AC.
6. Serum titer is the dilution following the last 4+ reaction. Interpretations for the screen test are listed in Table 4.

Table 3. Reagent Controls for the CF Test

Well No.	Purpose of Control	VBD	AG	C′	SRBC′s	Normal RBC′s	Acceptable of hemolysis on controls
1	Ag anticomplementary	.025	.025	.025	.05		0
2	Ag-hemolysin-hemolytic	.05	.025		.05		4
3	C′ 5.5($C'H_{50}$) units	.05		.025	.05		0
4	C′ 2.75($C'H_{50}$) units	.05		(.025) (1:1)	.05		+/-
5	C′ 2.75($C'H_{50}$) units w/Ag	.025	.025	(.025) (1:1)	.05		+/-
6	Hemolysin-hemolytic	.075			.05		4
7	Saline-hemolytic	.10				.025	4
8	C′-hemolytic	.075		.025		.025	4
9	Ag-hemolytic	.075	.025			.025	4

Number 9 wells on one plate. These are the reagent controls for the test.

Table 4. Equivalence of Readings by Percent Hemolysis and Numerical Value

Percent Hemolysis	Interpretation	Dourine, Glanders at 1:5	B. caballi, B. at 1:5
0%	4 plus	Positive	Positive
25%	3 plus	Suspicious	Positive
50%	2 plus	Suspicious	Positive
75%	1 plus	Suspicious	Negative
Few cells remaining	Trace	Negative	Negative
100%	Negative	Negative	Negative

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APPENDIX

Equipment

Diluters, 0.025 ml
Pipette droppers, 0.025 and 0.05 ml
Microtitration plates
Go-No-Go-delivery testers, 0.025 ml
Shaking apparatus
Spectrophotometer
Matched cuvettes
Waterbaths, 37 C and 58 C
Sealing tape for plates
Tape roller
Reading mirror
pH meter

Reagents

Hemolysin (rabbit anti-sheep serum)
Complement
Antigen
Sheep blood
Cyanmethemoglobin standard and reagent

Chemicals

Sodium-5, 5-diethyl barbituate (DEA drug license required to purchase)
5,5-diethyl barbituric acid
Other common chemicals

Preparing Stock Buffer Solution

1. To a 2-liter volumetric flask, add 1,500 ml of distilled water.
2. Add 83.0 gm of NaCl to the flask.
3. Add 10.19 gm of Na-5, 5-diethyl barbituate.
4. Mix by swirling the flask until chemicals are completely dissolved.
5. Add 34.58 ml 1 N HCl, and mix by swirling.
6. Add 5.0 ml of stock magensium calcium chloride solution. (See procedure for preparation below).
7. Fill the flask to the 2-liter mark with distilled water, and mix by inverting the flask.
8. Check the pH:
   a. Prepare a 1:5 dilution by adding 1 ml of stock buffer solution to 4 ml of distilled water.
   b. Check the pH of the 1:5 dilution.
   c. If the pH is below 7.3 or above 7.4, discard the stock buffer solution, and prepare fresh.
9. Store the stock buffer solution in the refrigerator.

Preparing Stock $MgCl_2$ (1 molar) $CaCl_2$ (0.3 molar) Solution

1. Add 100 ml of distilled water to a 250 ml Erlenmeyer flask.
2. Add 20.3 gm of $MgCl_2.6H_2O$ to the flask.
3. Add 4.4 gm of $CaCl_1.2H_2O$.
4. Mix by swirling.
5 Store in refrigerator.

Preparing Gelatin Solution

1. Dissolve 10 gm Bacto Gelatin or equivalent in 100 ml distilled water.
2. Dispense 10 ml into screw capped test tubes.
3. Sterilize in an autoclave for 15 minutes at 121 C.
4. Store at 4 C.
5. Melt in a 56 C waterbath for use.
6. Alternate method for preparing gelatin for VBD:
   a. Add 1 gm gelatin to 100 ml distilled water.
   b. Heat water to boiling.
   c. Add 200 ml of Stock VBD.
   d. Dilute to 1 liter with distilled water.

Determining Volume of Undiluted Complement Needed

Use the formula below to determine the number of ml of undiluted C′ needed to prepare a required volume of C′ at a given dilution.

$$\text{Volume (ml) of Undiluted C′ needed} = \frac{\text{Volume (ml) desired}}{\text{Reciprocal of dilution of C′ determined in C′ titration}}$$

Example: 40 ml of 1:133 dilution of C′ are needed.

$$\text{Volume (ml) of undiluted C′ needed} = \frac{40 \text{ ml}}{133} = 0.3 \text{ ml}$$

Therefore, you would use 0.3 ml C′ plus 39.7 ml diluent.

Preparing Hemoglobin Color Standards

1. Use a 5.0 ml pipette to add 3.0 ml of a well-mixed 2.0% RBC suspension to a test tube (15 x 125 mm).
2. Add 9.0 ml of distilled water, and shake the tube until all cells are lysed.
3. Add 3.0 ml of stock buffer solution (5X) to the tube.
4. Mix the hemoglobin solution thoroughly, and set it aside until it is needed.
5. Use a 5.0 ml pippette to add 3.0 ml of the 2.0% RBC suspension to a test tube (15 x 125 mm).
6. Add 12.0 ml of cold VBD to the tube with the 2.0% RBC's (0.4% RBC's).
7. Mix the 0.4% RBC suspension, and set it aside.
8. Label 11 serologic tubes (13 x 100 mm or size to be used in tests) with the percentages of hemolysis shown in the table below. Label the 0% standard with the date and time of preparation.
9. Use a 2.0 ml pipette to add 0.4% RBC suspension in the amounts shown in the table below to each tube.
10. Use a 2.0 ml pipette to add 0.4% RBC suspenion in the amounts shown in the table below.
11. Mix the standards by shaking the rack.
12. Centrifuge the tubes at 600 x g for 5 minutes.
13. Remove the tubes from the centrifuge without agitation, and store them in the refrigerator until they are needed.

Preparation of Color Standard

	Percent Hemolysis												
Reagents, ml	0	10	20	25	30	40	50	60	70	75	[illegible]	90	100
Hemoglobin solution	0	0.2	0.4	0.5	0.6	0.8	1.0	1.2	1.4	1.5	[illegible]	1.8	2.0
0.4%	2.0	1.8	1.6	1.5	1.4	1.2	1.0	0.8	0.6	0.5	0.4	0.2	0

Sheep Erythrocytes

Sheep blood, drawn aseptically, is preserved at 2-4 C in an equal volume of sterile Alsever's solution. Sheep blood preserved in this manner is allowed to age for 4 days prior to use.

After this stabilization period, the susceptibility of the erythrocytes to lysis by antibody and complement remains uniform for about 1 month, and a batch of blood may be used during this entire period, provided gross microbial contamination is avoided.

Preparing Alsever's Solution

1. To a 2-liter flask, add 1,200 ml of distilled water.
2. Add 24.6 gm of glucose.
3. Add 9.6 gm sodium citrate (dihydrate).
4. Add 5.04 gm sodium chloride.
5. Mix by swirling the flask until chemicals are completely dissolved.
6. Adjust pH to 6.1 with citric acid.
7. Sterilize by filtration through glass filter.

Preparation of Cyanmethemoglobin Reagent

1. Dilute 1 vial or tablet of reagent in twice the volume recommended on the label. This helps to overcome the "resistant cell phenomenon."
2. Store this reagent in a brown bottle (glass or polyethylene), or store in the dark at room temperature. (Do not use rubber or cork stoppers unless they are covered with parafilm as there is a chemical reaction between the cyanide and these materials which results in contamination of the reagent.
3. Discard the reagent if it becomes cloudy or if a precipitate forms after prolonged use.

Preparation of Cyanmethemoglobin Color Standards and Calculation of Target Optical Density (Absorbance)

1. Label 5 cuvettes for standards 72, 54, 36, 18, and 0 mg%.
2. Add to the tubes the volumes of 72-mg%[a] standard and cyanmethemoglobin reagent shown in the table below:

	Tube No. 1	Tube No. 2	Tube No. 3	Tube No. 4	Tube No. 5
Cyanmethemoglobin concentration (mg%)	72	54	36	18	0 (blank)
Volume of 80-mg% standard (ml)	4	3	2	1	0
Volume of cyanmethemoglobin reagent (ml)	0	1	2	3	4

3. Mix 5 cork stoppers in Parafilm-"M"®, and plug each cuvette. (Parafilm-"M"® is manufactured by Marathon Products, Neenah, Wisconsin).
4. Mix by inverting each cuvette.
5. Wipe each cuvette with a tissue to remove fingerprints; then read and record the optical density (OD) for each standard at 540 mu.
6. Take the sum of the milligram percent cyanmethemoglobin concentrations of all the standards (180 mg%).
7. Take the sum of the OD readings of all the standards.

[a] If concentration of hemoglobin standard is given in g/dl, it can be converted to mg% by multiplying g/dl by 4. (i.e. 18 g/dl x 4 = 72 mg%)

8. To calculate the factor, divide the sum of the concentrations by the sum of the OD readings. (See Example).

Concentration of standard (mg%)	OD 540 Reading of standards
80.0	0.460
60.0	0.350
40.0	0.240
20.0	0.120
0.0	0.000
180.0	1.170

$$\text{Factor} = \frac{180.0 \text{ mg\%}}{1.170 \text{ (total absorbancy)}} = 153.85 \text{ mg\% OD}$$

9. Use the factor to calculate the Target OD of your intrument for a 2.0% sheep cell suspension. Use the formula below:

$$\text{Target OD} = \frac{25.03 \text{ mg\%}}{\text{factor}}$$

Example: $\text{Target OD} = \frac{25.03}{153.85} = 0.16$

Sheep RBC (%)		Milligram Percent Cyanmethemoglobin
2.0	=	25.03
2.8	=	35.04
3.0	=	37.54

Commercial sources of certified cyanmethemoglobin standard and reagent:

Metrix hemoglobin standard and diluent tablets from:

Clinical and Diagnostics Division
Armour Pharmaceutical Company
530 E. 31st Street
Chicago, IL 60616

Hycel cyanmethemoglobin reagent and standard from:

Hycel, Inc.
P.O. Box 36329
Houston, TX 77036

Hemoglobin standard:

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178-9916